PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/24, G01N 33/569
C12N 5/00

A1

(11) International Publication Number: **WO 91/16452**

(43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/US91/02789

(22) International Filing Date: 23 April 1991 (23.04.91)

(30) Priority data:
513,057 23 April 1990 (23.04.90) US

(71) Applicant: CELLPRO INCORPORATED [US/US]; 22322 20th Avenue S.E., Bothell, WA 98021 (US).

(72) Inventor: BERENSON, Ronald, J. ; 6127 84th Avenue S.E., Mercer Island, WA 98040 (US).

(74) Agents: McMASTERS, David, D. et al.; Seed & Berry, 6300 Columbia Center, Seattle, WA 98104-7092 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR ENRICHING FETAL CELLS FROM MATERNAL BLOOD

(57) Abstract

A method is provided for enriching fetal nucleated, erythroid cells from maternal blood comprising the steps of: (a) incubating a sample of maternal blood with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of the ligand to the cells; (b) removing unbound blood products; and (c) incubating the bound cells in the presence of erythropoietin such that fetal cells are preferentially enriched.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CS	Czechoslovakia
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GN	Guinea
GR	Greece
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
PL	Poland
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

Description

A METHOD FOR ENRICHING FETAL CELLS FROM MATERNAL BLOOD

Technical Field

The invention relates to a method of enriching fetal cells from maternal blood.

Background of the Invention

Approximately 5.3 million women become pregnant in the United States yearly, resulting in 3.8 million deliveries. There are an additional 10 million deliveries in the other affluent countries in the world. Prenatal testing is used in a subgroup of these women who have a significant risk of having a fetus with a genetic disorder such as Down's Syndrome.

At present, the only way of diagnosing fetal disorders is to obtain cells from the amniotic fluid (amniocentesis) or the surface of the fetal sac (chorionicvillus) of the mother. These procedures are expensive and carry a risk of spontaneous abortion of between ½%-1%. Because of the risk of spontaneous abortion, these collection procedures are recommended only for women who are at a high-risk of carrying a child with a genetic defect. For example, only women over age 35 are advised to have the test because the risk of Down's Syndrome is higher in that group. Some of these women refuse the test because of the risk of spontaneous abortion. Even though many of these high-risk women are tested, only a fraction of fetuses with Down's Syndrome are detected. The high-risk women represent such a small portion of the women having children that the low-risk population still delivers most of the afflicted children. Eighty percent of those children born with Down's Syndrome are from the "low-risk," under 35-year-old group. This situation is also true of many other genetic defects or disorders.

It is therefore desirable to provide a test that would resolve this testing dilemma by providing a safe method which could be available to all pregnant women, irrespective of risk factor and without risk of spontaneous abortion. Although it is known that fetal cells circulate in the blood stream of pregnant women (see Kulozik and Pawlowitzki, "Fetal Cells in the Maternal Circulation: Detection by Direct AFP-Immunofluorescence," Human Genetics 62:221-224, 1982), fetal cells are present in such low concentrations that the procedures necessary to isolate them are extremely difficult and time-consuming.

For example, Hertzenberg et al. ("Fetal Cells in the Blood of Pregnant Women: Detection and Enrichment by Fluorescence-Activated Cell Sorting," Proc. Natl. Acad. Sci., USA 76:1453-1455, March 1979) used a Fluorescence-Activated Cell Sorter (FACS) in order to detect fetal cells in maternal blood. The procedure used, however, is not adaptable to routine clinical testing procedures, in part, due to the great expense and expertise required to run a FACS machine. The method of Hertzenberg et al. is also deficient for routine clinical testing because it requires determination of HLA types. Bianchi et al. ("Direct Hybridization to DNA From Small Numbers of Flow-Sorted Nucleated Newborn Cells," Cytometry 8:197-202, 1987) also used a FACS machine to detect nucleated cells, although the blood sample was not obtained from the mother but from the newborn's umbilical cord.

The present invention provides a method for enriching fetal cells that overcomes these disadvantages, and further provides other related advantages.

Summary of the Invention

The present invention is directed toward methods for enriching fetal nucleated, erythroid cells from maternal blood. Within one aspect of the present invention, such a method comprises the steps of: incubating a sample of maternal blood with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of the ligand to the cells; removing unbound blood products; and incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched. Within one embodiment of this aspect of the invention, the immobilized ligand is an immobilized antibody.

Within another aspect of the invention, a method is provided for enriching fetal nucleated, erythroid cells from maternal blood, comprising the steps of: incubating a sample of maternal blood with a first member chemically linked to a ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of the ligand to the cells; adsorbing the cells to an immobilized second member, the second member being capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; removing unbound blood products; and incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched. Suitable first member-second member binding pairs include biotin-avidin, biotin-streptavidin, biocytin-avidin, biocytin-streptavidin,

methotrexate-dihydrofolate reductase, 5-fluorouracil-thimydylate synthetase, and riboflavin-riboflavin binding protein. Within one embodiment of this aspect of the invention, the first member which is chemically linked to a ligand is a biotinylated antibody and the immobilized second member is immobilized avidin.

Within another aspect of the present invention, the method comprises the steps of: incubating a sample of maternal blood with a first ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of the first ligand to the cells, incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand; adsorbing the cells to an immobilized second member, the second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; removing unbound blood products; and incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched. Within one embodiment, the first ligand is an antibody which specifically binds to fetal nucleated, erythroid cells. Within preferred embodiments, the first member which is chemically linked to the second ligand is a biotinylated antibody. In such an embodiment, the immobilized second member is immobilized avidin.

As an alternative to the step of incubating bound cells in the presence of erythropoietin, another aspect of the present invention comprises the steps of: (a) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and (b) incubating the bound cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs. In addition, the present invention may also be performed using a combination of both erythropoietin enrichment and the method described above wherein the cells are incubated with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells, followed by the incubation of bound cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs. These two enrichment methods may be performed sequentially, and in either order.

Within another aspect of the present invention, a method is provided for enriching fetal nucleated, erythroid cells from maternal blood, comprising the steps of: incubating a sample of maternal blood in the presence of

erythropoietin such that the fetal cells are enriched; incubating the enriched cells with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of the ligand to the cells; and removing unbound blood products. Alternatively, within another embodiment of the present invention, the enriched cells may be immobilized by incubating them with a first member chemically linked to a ligand capable of specifically binding to the cells under conditions and for a time sufficient to allow specific binding of the ligand to the cells, and adsorbing the cells to an immobilized second member, the second member being capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1} . Within yet a further embodiment of the present invention, the enriched cells may be immobilized by incubating the enriched cells with a first ligand capable of specifically binding to the cells under conditions and for a time sufficient to allow specific binding of the first ligand to the cells; incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand; and adsorbing the cells to an immobilized second member, the second member being capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}. Suitable combinations of first and second ligand are discussed in detail below.

In addition, within the aspects discussed above, the methods may further comprise (subsequent to removing the unbound blood products) the steps of: incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide, such that selective hemolysis of maternal blood cells occurs.

Within other aspects of the present invention, as an alternative to first incubating a sample of maternal blood in the presence of erythropoietin, the cells are incubated with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells, followed by incubation of the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide, such that selective hemolysis of maternal blood cells occurs.

Within the present invention, a variety of ligands may be utilized, including antibodies, erythropoietin, and transferrin. The ligand may be immobilized on any of a variety of solid supports, such as hollow fibers, beads,

magnetic beads, plates, dishes, flasks, meshes, screens, solid fibers, membranes, and dipsticks.

Within another aspect of the present invention a method is provided for typing chromosomes of fetal nucleated erythroid cells, comprising the steps of: incubating the fetal nucleated erythroid cells in a media containing erythropoietin under conditions and for a time sufficient to induce metaphase in the cells; fixing the DNA of the cells; staining the fixed DNA such that chromosomes may be observed; and examining the stained DNA thereby allowing the typing of the chromosomes.

These and other aspects of the present invention will become evident upon reference to the following detailed description.

Detailed Description of the Invention

The present invention provides methods for the enrichment of fetal nucleated, erythroid cells from maternal blood. Maternal blood contains, among many other types of cells, both adult and fetal nucleated, erythroid cells. Through the efforts of the present invention, fetal nucleated, erythroid cells may be enriched from as few as 1 in 10^6 in maternal blood, to an enriched concentration of about 1 in 10^3, and preferably, to a out 1 in 10^2. Within the context of the present invention, nucleated erythroid cells contain a nucleus and generally include erythroblasts as well as other erythroid precursor cells.

Maternal blood may be obtained from a pregnant female using conventional techniques well known in the art. Preferably, peripheral blood is drawn from an easily obtainable source such as the antecubital vein (the arm vein) with conventional venipuncture techniques. Once the maternal blood has been drawn, it may be frozen using conventional techniques, or stored at 4°C for a maximum of 4 to 7 days. Various anticoagulants may be added to the blood as necessary, including, among others, ACD, CPDA, EDTA, and Heparin.

The maternal blood is then subjected to a selection method in accordance with the present invention, wherein preferentially enriched fetal cells may be obtained without the need for further purification, for example, by a Fluorescence-Activated Cell Sorter (FACS). In general, the methods of the present invention comprise the steps of: (1) incubating the maternal blood with either an immobilized ligand, or a ligand which will subsequently be immobilized, such that the ligand binds to and hence immobilizes the fetal nucleated, erythroid cells; (2) removing unbound blood products; and (3) preferentially enriching the bound cells for fetal nucleated, erythroid cells. As noted above, these basic steps

may be performed in an alternative order, for example, comprising the steps of: (1) preferentially enriching maternal blood for fetal nucleated, erythroid cells; (2) incubating the enriched cells with either an immobilized ligand, or a ligand which will subsequently be immobilized, such that the ligand binds to and hence immobilizes the fetal nucleated, erythroid cells; and (3) removing unbound blood products. The present invention may be performed utilizing a device as described in a related application entitled "Immunoselection Device and Method", attorney's docket number 200072.401, which is incorporated herein by reference.

Within one aspect of the present invention, the maternal blood is incubated with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions, and for a time sufficient to allow binding of the cells to the ligand. Generally, incubation of about 15 to 30 minutes at 4°C to 37°C is preferred. If the incubation step occurs as the cells are passed over a column, the flow rate should be sufficiently slow to allow the cells to bind. Preferably, the cells should be allowed at least 15 minutes in which to traverse the bed of the column.

As noted above, the ligand should be chosen such that it is capable of specifically binding fetal nucleated, erythroid cells. Within the context of the present invention, the ligand is defined to be "specifically binding" if it is capable of binding to fetal nucleated, erythroid cells, but not to more than about 10% of the maternal blood cells. The relative percentage of bound fetal nucleated, erythroid cells to other cells may be readily determined by analysis with a specific marker such as Alpha Fetal Protein (AFP). For example, at any point within the methods discussed below, the relative percentage of fetal cells to maternal cells may be determined with either glucose oxidase or fluorescein conjugated anti-AFP antibody (see A. Kulozik and I. H. Pawlowitzki, "Fetal Cells in the Maternal Circulation: Detection by Direct AFP-Immunofluorescence," Human Genet. 62:221, 1982). This determination is preferably performed after two or more purification steps.

Ligands which specifically bind to fetal nucleated, erythroid cells are known in the art, including erythropoietin (Amgen, Thousand Oaks, Calif.), transferrin (Sigma Chemical Co., St. Louis, Mo.) and selected antibodies. Monoclonal antibodies which specifically recognize nucleated erythroid cells are particularly preferred. Monoclonal antibodies to nucleated erythroid cells such as anti-transferrin receptor antibodies may be obtained from conventional suppliers (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). Alternatively, monoclonal anti-erythroid antibodies, such as EP-1, may be

produced using techniques well known in the art (see Yokochi et al., "Monoclonal Antibodies Detecting Antigenic Determinants With Restricted Expression On Erythroid Cells: From the Erythroid Committed Progenitor Level to the Mature Erythroblast," Blood 63:1376, 1984; see also Heddy Zola (ed.), Monoclonal Antibodies: A Manual of Techniques, CRC Press, Boca Raton, Fla., 1987). Briefly, cells may be generated for immunization from fetal liver clonal erythroid cultures, and enriched for progenitor cells. Thus, the population of cells used for antigens and for primary screening may contain immature erythroblasts, erythroblasts of an intermediate degree of maturity, and presumably, progenitor cells of BFU-E and CFU-E types. These cells may be used for intravenous immunization, followed by removal of the spleen and fusion of the spleen cells with a myeloma line such as NSI, using standard techniques. The resulting fused cells, or hybridomas, may then be screened against the above-described cells using conventional techniques (see Yokochi et al., *supra*).

The entire specifically binding antibody need not be used as the ligand. More specifically, only the binding region of the antibody is necessary to specifically bind fetal nucleated, erythroid cells. Thus, antibody fragments such as Fab or $F(ab')_2$ fragments may be used within the present invention. Additionally, the binding regions of the specifically binding antibody may be incorporated into a new protein, which may be used as the ligand (see Reichmann et al., "Reshaping Human Antibodies For Therapy," Nature 332:323-327, 1988; Verhoeyen et al., "Reshaping Human Antibodies: Grafting an Antilysozyme Activity," Science 239:1534-1536, 1989; and Roberts et al., "Generation of an Antibody with Enhanced Affinity and Specificity for its Antigen by Protein Engineering," Nature 328:731-734, 1987).

Within the present invention, the ligand is immobilized in order to allow separation of bound cells from other blood products. Many suitable supports are well known in the art and include, among others, hollow fibers (Amicon Corporation, Danvers, Mass.), beads (Polysciences, Warrington, Penn.), magnetic beads (Robbin Scientific, Mountain View, Calif.), plates, dishes and flasks (Corning Glass Works, Corning, N.Y.), meshes (Becton Dickinson, Mountain View, Calif.), screens and solid fibers (see Edelman et al., U.S. Patent No. 3,843,324; see also Kuroda et al., U.S. Patent No. 4,416,777), membranes (Millipore Corp., Bedford, Mass.), and dipsticks. A variety of different sources exist for supports other than those designated. Particularly preferred is a support such as Biogel P-60™ (BIORAD, Richmond, Calif.). Biogel P-60™ is a porous polyacrylamide hydrogel bead. The beads are generally spherical, on average

about 250 microns in size, and have an average pore size which excludes molecules larger than approximately 60,000 daltons.

A variety of methods may be used to immobilize the ligand onto a support. For example, a ligand, such as an antibody, may be directly coupled to the support by various methods well known in the art (see J. K. Inman, Methods In Enzymology, Vol. 34, Affinity Techniques, Enzyme Purification: Part B, W. B. Jakoby and M. Wilchek (eds.), Academic Press, New York, p. 30, 1974; see also M. Wilchek, and W. Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications," Analyt. Biochem. 171:1-32, 1988). These methods include the use of glutaraldehyde, carbodiimide, carbonyl diimidazole, cyanogen bromide, tosyl chloride, biotin/avidin, and biotin/streptavidin. Once the ligand has been immobilized onto the support, the maternal blood may be incubated with the immobilized ligand under conditions, and for a time sufficient to allow binding of the ligand to the cells. Within the context of the present invention, suitable conditions for binding to occur include incubation in a physiological buffer at about 4°C to about 37°C. Particularly preferred temperatures range from about 4°C to room temperature. The time of incubation depends on the affinity and avidity of the ligand for the cell, and may be readily determined. Generally, incubation for about 15 minutes to one hour is preferred. Following incubation, unbound blood products may be removed, and fetal cells enriched using the methods described herein.

Within another aspect of the present invention, a sample of maternal blood is incubated under suitable conditions with a ligand which is chemically linked to a first member, and then adsorbed to a second member which is immobilized on a solid support. The first member should be capable of binding to the second member with an affinity of greater than about 10^8 M^{-1}. Many suitable first member-second member binding pairs are well known in the art. These include, among others, biotin-avidin, biotin-streptavidin, biocytin-avidin, biocytin-streptavidin, methotrexate-dihydrofolate reductase, 5-fluorouracil-thimydylate synthetase, riboflavin-riboflavin binding protein, antibody-protein A, and antibody-protein G. In a preferred embodiment, the first member is biotin and the second member is avidin.

Either member of the above described binding pairs may function as the second member, with the complementary member functioning as the first member. Furthermore, combinations of the first member - second member binding pair may be employed. For example, biotin may be linked to the ligand, as well as adsorbed to the support. The cell, ligand, biotin-complex and biotin,

support-complex may then be bound together through an incubation step with avidin. Avidin is multivalent, permitting the formation of a cell, ligand, biotin, avidin, biotin, support-complex which immobilizes the cell.

Within one example of this embodiment, a sample of maternal blood is incubated with a biotinylated antibody under conditions and for a time sufficient to allow binding to occur. The sample is then incubated with, or passed over a support which contains immobilized avidin. Cells which are coupled to the biotinylated antibody are adsorbed to the immobilized avidin, thus allowing separation of cells from unbound blood products. Subsequently, unbound blood products may be removed, and fetal cells enriched using methods described below.

Within yet another aspect of the present invention, a two-step method is used to immobilize the fetal nucleated, erythroid cells. Briefly, a first ligand is incubated with a sample of the maternal blood under suitable conditions as described above. Subsequently, a second ligand which has been chemically linked to a first member is added. The second ligand is capable of binding to the first ligand. The cell, first ligand, second ligand, first member-complex may then be adsorbed onto an immobilized second member, thus allowing the separation of cells from unbound blood products. Representative examples of the first member-second member binding pair have been discussed above. Representative examples of the first ligand include erythropoietin, transferrin, and selected antibodies. Once the first ligand has been selected, the second ligand is chosen such that it specifically recognizes and binds to the first ligand. Within a preferred embodiment, the second ligand is an antibody, for example an anti-erythropoietin (Terry Fox Laboratory, Vancouver, B.C., Canada), anti-transferrin (Chemicon Intl., Inc., Temecula, Calif.), or anti-immunoglobulin antibody. Anti-immunoglobulin antibodies may be prepared using techniques well known in the art, or may be obtained from conventional sources, including, among others, Sigma Chemical Co., St. Louis, Mo., and Becton Dickinson Immunocytometry Systems, Mountain View, Calif.

Within a preferred embodiment, the first ligand is an antibody which specifically recognizes fetal nucleated, erythroid cells such as an anti-transferrin receptor antibody (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). The antibody is incubated with a sample of maternal blood. A biotinylated anti-immunoglobulin antibody, such as biotinylated goat anti-mouse IgG (the second ligand which is chemically linked to a first member) is then added and incubated with the sample. The sample is then incubated with, or passed over a bed of material which contains the immobilized second member, in

this case, immobilized avidin. The cell, antibody, anti-immunoglobulin antibody, and biotin-complex will be adsorbed to the immobilized avidin, thus allowing the subsequent removal of unbound blood products.

As noted above, once the cells have been immobilized, unbound blood products may be removed. In one embodiment, the immobilized cells are rinsed with a physiological buffer, thereby removing the unbound blood products. Various methods may be used to rinse the immobilized cells, depending upon the type of support chosen. These methods include, among others: washing or flushing the support; magnetically attracting the support out of solution, followed by resuspension in a physiological buffer; and centrifugation followed by resuspension. Various physiological buffers are also well known in the art, including PBS, PBS plus albumin, such as Bovine Serum Albumin (BSA), normal saline, and cell culture media.

Once unbound blood products have been removed, bound cells may be preferentially enriched for fetal nucleated, erythroid cells. As noted above, at least two alternative methods may be used either separately, or together. If the two methods are performed together, either method may be performed first. Within one embodiment the bound cells are cultured under selected culture conditions in the presence of erythropoietin (Amgen, Thousand Oaks, Calif.) (see Emerson et al., "Developmental Regulation of Erythropoiesis by Hematopoietic Growth Factors: Analysis on Populations of BFU-E From Bone Marrow, Peripheral Blood and Fetal Liver," Blood 74(1):49-55, 1989; see also Linch et al., "Studies of Circulating Hemopoietic Progenitor Cells in Human Fetal Blood," Blood 59(5):976-979, 1982). Selected culture conditions generally include growth in standard cell culture media, without any other cytokines other than erythropoietin. This preferentially allows fetal, but not maternal, nucleated erythroid cells to grow. Particularly preferred media includes Iscoves' Modified Dulbecco's Medium (Gibco, Grand Island, N.Y.) containing a final concentration of 20% fetal bovine serum, and 2 U/ml purified urinary human EPO.

Within the second method, fetal nucleated, erythroid cells are preferentially enriched based upon their uptake of ammonium ions and the selective hemolysis of maternal blood cells, or more specifically, of maternal erythroid cells (see generally Jacobs and Stewart, "The Role of Carbonic Anhydrase in Certain Ionic Exchanges Involving the Erythrocyte," J. Gen. Physiol. 25:539-552, 1942; and Maren and Wiley, "Kinetics of Carbonic Anhydrase in Whole Red Cells as Measured by Transfer of Carbon Dioxide and Ammonia," Molecular Pharmacology 6:430-440, 1970). Briefly, the cells are incubated in the

presence of ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the cells. This generally takes approximately 5 to 30 minutes. The cells are then incubated in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

Within the context of the present invention, many compounds may provide suitable sources of ammonia, chloride ions, and carbon dioxide. For example, suitable sources of ammonia include, among others, ammonia and the ammonium salts. Suitable sources of chloride ions include, among others, NaCl, KCl, $MgCl_2$, and $CaCl_2$. Suitable sources for carbon dioxide include, among others, carbon dioxide in solution, carbonate, and bicarbonate.

In addition, many carbonic anhydrase inhibitors are known in the art, including, among others, most monovalent anions such as cyanide and cyanate, monovalent sulfides, sulfonamides, and acetazolamide (see Lindskog et al., The Enzymes 5:587, 1971; Ward and Cull, Arch. Biochem. Biophys. 150:436, 1972; and Pocker and Watamori, Biochem. 12:2475, 1973). The carbonic anhydrase inhibitors should be selected so as to function under physiological conditions. Preferred carbonic anhydrase inhibitors include sulfanilamide and acetazolamide.

Bound cells may be released by various methods either subsequent to, or prior to enrichment. If bound cells are released prior to enrichment, they may be subsequently enriched using the above-described methods. Various methods are known in the art for releasing cells. Within one such method, cells may be cultured with or without cytokines. Cytokines, such as IL-2, can cause proliferation of cells, or changes in the surface characteristics of the cells, such that the cells or their progeny are released from a support. Within another method, cleavage of the ligand or of the cell-ligand bond may release the cell. Various cleavable ligands and cleaving enzymes are known in the art, including among others, papain and trypsin. Within yet another method, the cells may be released by mechanical, gravitational, or electromagnetic forces. A particularly preferred method is mechanical agitation, for example, by agitation of the beads through pipetting, stirring, shaking, vibration, or sonication.

Within another aspect of the present invention, a method is provided wherein the cells are first enriched, followed by adsorption of fetal nucleated, erythroid cells and removal of unbound blood products. Briefly, fetal cells may be enriched by first incubating maternal blood with ammonia and chloride ions, and a carbonic anhydrase inhibitor. After the cells have been incubated under conditions and for a time sufficient to allow accumulation of

ammonium ions, the cells are treated with ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs. Fetal cells may also be enriched by incubation with erythropoietin as discussed above, or by a combined treatment using both erythropoietin and the method discussed above wherein the cells are treated with ammonia and carbon dioxide. Furthermore, as discussed above, the two methods may be performed in either order.

The enriched cells remaining after the method(s) described above may then be immobilized using any of the above discussed methods, including: (1) an immobilized ligand which specifically binds to fetal nucleated, erythroid cells remaining after enrichment; (2) incubating cells remaining after enrichment with a first member linked to a ligand, the ligand being capable of specifically binding to fetal nucleated, erythroid cells, followed by adsorption of the cells to an immobilized second member, the second member being capable of binding to the first member with an affinity constant of greater than about $10^8 M^{-1}$; and (3) incubating cells remaining after enrichment with a first ligand, followed by incubation with a second ligand which is chemically linked to a first member which is capable of binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand, followed by adsorption of the cells to an immobilized second member, the second member being capable of binding to the first member with an affinity constant of greater than about $10^8 M^{-1}$.

Subsequent to immobilization of the cells, unbound blood products may be removed using methods described above. If the cells were initially enriched using erythropoietin, subsequent to removing unbound blood products, the cells may be treated using the method wherein ammonia and carbon dioxide are used. Similarly, if these cells were initially treated with the method wherein ammonia and carbon dioxide are used, subsequent to removing unbound blood products, the cells may be treated with erythropoietin.

Fetal cells which have been enriched from maternal blood may be characterized by incubating the preferentially enriched cells with a marker capable of selectively binding to fetal cells. A marker selectively binds to fetal cells when its presence on the target cells is greater than 10-fold more than the quantity on maternal cells. Various markers are known in the art, including, for example, antibodies to Alpha Fetal Protein ("AFP") (see Kulozik et al., "Fetal Cells in the Maternal Circulation: Detection by Direct AFP-Immunofluorescence," Human Genet. 62:221-224, 1982), or antibodies to antigen "i" (see Y.W. Kan et al., "Concentration of Fetal Red Blood Cells From a Mixture

of Maternal and Fetal Blood by Anti-i Serum," Blood 43(3):411-415, 1974). Antibodies to the fetal cell marker may be labeled using techniques well known in the art, and used to detect the presence of fetal cells.

Within a preferred embodiment of the present invention, ligand is removed from the cell surface through the method described in a related application entitled "Methods for Removing Ligands from a Particle Surface," attorney's docket number 200072.403, which is incorporated herein by reference. Such removal may be particularly advantageous prior to characterization, as discussed above.

As described herein, enriched fetal cells have a variety of uses. For example, through *in situ* hybridization the presence of a selected genetic material may be detected in a fetal cell. Similarly, DNA or RNA amplification may also be used to detect a selected genetic sequence in fetal cells. The methods of the present invention are particularly useful for fetal cells which are enriched to such an extent that they are amenable to chromosomal typing by conventional cytogenetics techniques wherein spreads of the cells' chromosomes are examined under a microscope.

In situ hybridization may be used as a method for detecting the presence of a selected genetic material within cells (see Pinkel et al., Proc. Natl. Acad. Sci., USA 85:9138-42, 1988; see also Hopman et al., "Detection of Numerical Chromosome Aberrations in Bladder Cancer by *In Situ* Hybridization, Am. J. of Path." 135(6):1105-1117, 1989). Briefly, the genetic material associated with the preferentially enriched cells is first exposed using techniques well known in the art. The genetic material is then incubated with a labeled probe capable of specifically hybridizing to the genetic material, under conditions and for a time sufficient to allow hybridization to occur (see, for example, K. E. Davies, Human Genetic Diseases, IRL Press, Oxford, U.K., 1986). Finally, the presence of the hybridized labeled probe is detected. Within a preferred embodiment, the genetic material is denatured after the step of exposing. Within the context of the present invention, genetic material includes whole chromosomes, DNA, and RNA.

Various methods are also well known in the art for amplifying and detecting genetic material. For example, if present, a selected genetic sequence may be amplified using techniques well known in the art, and then probed for presence of that sequence (see Kogan et al., "An Improved Method For Prenatal Diagnosis of Genetic Diseases by Analysis of Amplified DNA Sequences," The New Eng. J. of Med. 317(16):985-990, 1987; see also Witt and Erickson, "A Rapid Method for Detection of Y-Chromosomal DNA from Dried Blood Specimens by

the Polymerase Chain Reaction," Human Genet. 82:271-274, 1989). Methods for amplification include Polymerase Chain Reaction ("PCR") (see Mullis et al., U.S. Patent No. 4,683,195; Mullis et al., U.S. Patent No. 4,683,202; and Mullis et al., U.S. Patent No. 4,800,159, which are incorporated herein by reference), and RNA-based amplification techniques (see Lizardi et al., Bio/Technology 6:1197-1202, 1988; Kramer et al., Nature 339:401-402, 1989; and Lomeli et al., Clinical Chemistry 35(9):1826-1831, 1989; see also Kramer et al., U.S. Patent No. 4,786,600, which is incorporated herein by reference).

PCR is the most commonly used method for amplifying DNA sequences. Briefly, amplification entails adding the appropriate primer(s), enzymes and nucleotides into a reaction mixture, followed by several (20-80) cycles of denaturation and annealing in order to amplify the small amount of target DNA. The DNA mixture is then separated by electrophoresis and hybridized with a labeled probe to detect the presence of the target sequence of DNA.

The preferentially enriched fetal cells may also be chromosomally typed (see Human Cytogenetics, D.E. Rooney and B.H. Czepulkouski (eds.), IRL Press, Oxford, U.K., 1986). Briefly, within a preferred embodiment, a sample containing at least 1 fetal cell in 10^5 other cells is cultured for 2-6 days in Iscoves' Modified Dulbecco's Medium (IMDM) (Gibco, Grand Island, N.Y.) containing a final concentration of 20% fetal bovine serum (FBS) (HYCLONE™, Logan, Utah), and 2 U/ml of highly purified recombinant erythropoietin (Terry Fox Laboratory, Vancouver, B.C., Canada), in order to increase the proportion of cells in metaphase. The cells are then incubated in colcemid, fixed, attached to microscope slides, trypsin-treated, and stained with Wright's stain. The slides may then be scanned microscopically for abnormal chromosomes.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Example 1
Carboxylation of a Polyacrylamide Gel

Seventeen grams of dry Biogel P-60™, (50-100 mesh (wet), coarse beads) (BIORAD, Catalog No. 150-1630, Richmond, Calif.) are added to 1.5 l of 0.5 M $NaHCO_3$/0.5 M Na_2CO_3. The pH is adjusted to 10.5 with NaOH and

carefully stirred with a mixer (RZR1, Carfamo, Wiarton, Ontario, Canada) so as not to damage the beads for approximately 20 to 30 minutes. The mixture is then placed in a 60°C water bath. After the mixture reaches a temperature of 60°C, it is incubated for an additional 2 hours (at 60°C) with occasional stirring. The mixture is then removed from the water bath, and placed in an ice bath to bring the mixture temperature down to room temperature.

The beads are washed several times with distilled or deionized water, followed by several washings of PBS using a coarse glass filter connected to a vacuum. The carboxylated gel may be stored in PBS at 4°C, and is stable for up to one year if sterilized or stored with a preservative.

Example 2

Avidin Conjugating the Carboxylated Biogel

PBS is first removed from a measured amount of carboxylated Biogel by filtering with a coarse glass filter connected to a vacuum. The gel is then equilibrated in distilled or deionized water for 15-30 minutes. Equilibration in water causes an expansion of the gel to a volume of about 4 times it's previously measured amount. The gel is resuspended in 10 ml of distilled or deionized water for each ml of gel (as originally measured in PBS).

Twenty mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-HCl) (Sigma Chemical Co., Catalog No. E7750, St. Louis, Mo.) is added for each ml of gel as originally measured. The pH is rapidly adjusted to 5.5 by dropwise addition of HCl. Care is taken to maintain the pH at 5.5; pHs of less than 5.0 or greater than 6.0 result in significantly less activation of the Biogel. The mixture is stirred for five minutes.

Avidin (International Enzymes, Inc., Fallbrook, Calif.) is dissolved at a concentration of between 10 and 100 mg/ml in deionized water. Next, 500 μg of avidin is rapidly added for each ml of gel (as originally measured in PBS). The mixture is stirred for 1.5 hours. Next, 2 M glycine is added to give a final concentration of 0.2 M glycine in the mixture, and stirred for an additional 1 hour.

The gel is washed with several volumes of PBS using a coarse glass filter and vacuum, and stored in PBS with 0.1% NaN_3 at 4°C. The gel is stable for approximately one year.

Example 3

Immunoadsorption of Maternal Cells With The Two-Step Method

Preparation of Cells

Twenty milliliters of blood is obtained from a pregnant female and suspended in an equal volume of PBS with 1% Bovine Serum Albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.), in four 50 ml centrifuge tubes. The number of nucleated cells/ml is counted in the following manner: A 50 μl aliquot of mixed, anticoagulated whole blood is diluted into 3 ml of a 3% acetic acid solution. After vortexing, 7 μl samples of diluted blood are loaded into each of two chambers of a hemacytometer. After about 3 minutes to allow settling of the cells, the nuclei of cells, which are almost all lysed by the acetic acid, are counted in the four ruled fields per chamber of a hemacytometer with improved Neubauer rulings (VWR Scientific, San Francisco, Calif.), each field representing 0.1×10^{-3} μl sample volume. The average number of nuclei per field is multiplied by a dilution factor of 61×10^4 to calculate the number of nucleated cells per milliliter of whole blood. If the average is less than 10 nuclei per field, the procedure is repeated except 50 μl blood is diluted into only 1 ml acetic acid, with a resulting new dilution factor of 21×10^4.

Each tube of diluted blood is underlayered with 5 ml of Histopaque 1077 (Sigma Chemical Co.) and centrifuged at 700 g for 15 minutes at room temperature. Cells at the interface are collected and washed once in PBS plus 1% BSA. The pellet is resuspended in 100 μl in PBS plus 1% BSA.

Twenty micrograms/milliliters of anti-transferrin receptor antibody (Becton Dickinson, Immunocytometry Systems, Mountain View, Calif.) is added to the mixture and incubated for 15 minutes on ice. The cells are then washed once with 4 ml of PBS plus 1% BSA and centrifuged at approximately 400 g for 5 minutes.

The cells are then gently resuspended to 1 ml and 1 μg/ml of biotinylated goat anti-mouse IgG (Southern Biotech, Birmingham, Ala.) is added. The mixture is incubated for 15 minutes on ice and washed twice as described above with PBS plus 1% BSA.

Preparation of Columns

Carboxylated Biogel P-30™ (prepared as described above) is allowed to equilibrate to room temperature and placed in a K9/15 column (Pharmacia, Piscataway, N.J.) to a total bed height of 1 cm. The column is washed with PBS, followed by washes with PBS plus 5% BSA. This column functions as a "pre-column." The avidin column contains avidin-conjugated Biogel P-60™, which is

prepared as described above. The avidin-conjugated Biogel is allowed to equilibrate to room temperature, and placed in a K9/15 column to a total bed height of 4 cm. The column is then washed with several volumes of PBS, followed by washes with PBS plus 5% BSA.

Immunoadsorption of Cells

Cells which have been prepared as described above are resuspended in PBS plus 5% BSA to a volume of 1 ml. The cells are then gently transferred onto the top of the gel bed of the pre-column filter. The cells are allowed to filter through the pre-column and are washed with 1 ml PBS plus 5% BSA. A peristaltic pump (Cole-Parmer, Rockford, Ill.) controls flow from the avidin column to a rate of about 1 ml/minute. Once the cells have almost run down to the top of the avidin column bed, 1-2 ml of PBS plus 5% BSA is added to the top of the avidin column in order to wash out remaining cells. The column is washed out with 4-6 ml of PBS plus 5% BSA, followed by 4-6 ml of PBS.

Removal of Adsorbed Cells From The Avidin Column

The avidin column is placed on top of a 15 ml centrifuge tube. The valve of the column is opened and 15 ml of RPMI 1 is added to the column with a wide bore, 9-inch transfer pipette. The RPMI is added to the column while the pipette is used for mechanically agitating and resuspending the cell bed, thus allowing cells to become detached from the gel matrix, and to filter into the centrifuge tube. The tube is then centrifuged at 400 g for 5 minutes and resuspended in cell culture media as described below.

Example 4

Preferential Enrichment of Fetal Nucleated, Erythroid Cells

Enrichment of Fetal Cells With Erythropoietin

Cells which are separated from the Avidin-Biogel column above, are resuspended in Iscoves' Modified Dulbecco's Medium (IMDM) (Gibco, Grand Island, N.Y.) containing a final concentration of 20% fetal bovine serum (FBS) (HYCLONE™, Logan, Utah), and 2 U/ml of highly purified recombinant erythropoietin (Terry Fox Laboratory, Vancouver, B.C., Canada). Cells are diluted to 5×10^6 nucleated cells/ml and 200 μl is plated into each well of a 96 well tissue culture plate with round bottoms (Corning Glass Works, Corning, N.Y.).

Enrichment of Fetal Cells by Ammonium Ion Differentiation

Cells which are separated from the Avidin column above, are adjusted to a concentration of less than 2 X 10^7 nucleated cells/ml. One volume of the cell mixture is chilled, and a solution at 29°C containing 18 volumes of 0.1844 M NH_4Cl and 2 volumes of 10 μM acetazolamide is added. After 2 minutes, 2 volumes of 3mM NH_4HCO_3 is rapidly added, and the whole mixture gently stirred for 3 minutes. Cells are washed several times with PBS by centrifugation in order to remove cellular debris.

Example 5

In Situ Hybridization

Enriched cells are exposed to a hypotonic solution (.075 M KCl) for 12 minutes at 37°C. The tubes are inverted once during the incubation to keep the cells suspended. Twenty drops of freshly prepared fixative (3:1 methanol:acetic acid) is added to the cells, vortexed, and then centrifuged for 8 minutes at approxiamtely 250 g. Fresh fixative is added to the cells, followed by incubation for one hour at room temperature. The cells are centrifuged for 8 minutes at approximately 250 g. Fresh fixative is added and the process is repeated one more time. Finally, the cells are resuspended in a small amount of fixative and placed at 4°C overnight.

The next day the cells are vortexed and placed onto microscope slides (Baxter, McGaw Park, Ill.) which have been cleaned with ethanol and dipped in distilled water. The slides are allowed to dry for two days at room temperature.

The slides are heated in 70% formamide in 2x SCC (0.30 M NaCl, 0.030 M Na citrate) to 68°C-70°C for 2.5 minutes. The slides are then immediately placed in a rinse of 70% ethanol in water. Following the rinse, the slides are placed successively in 70%, 95%, and 100% ethanol solutions for 5 minutes each. Each alcohol solution must be maintained at ≤ -20°C. The slides are air dried.

A probe is prepared from plasmid DNA according to the method of Page et al ("Single coy sequence hybridizes to polymorphic and homologous loci on human X and Y chromosomes" PNAS 79:5352-5356, 1982), from American Type Culture Collection (ATCC) No 57261; except that biotin-dATP is incorporated into the probe. Twenty five microliters of the biotinylated probe (5 μg/ml) per slide is placed in a microfuge tube. The probe is heated to 70°C for 5

minutes then immediately placed on ice. Twenty microliters of the probe solution is placed onto each slide and covered with a 22 x 40 mm coverslip. The slides are placed into a box with a wet paper toweling liner and incubated at 37°C for 12-18 hours.

A 50% solution of formamide in 1 x SCC is warmed to 37°C. Coverslips are removed from the slides and immersed into the 50% formamide solution for 30 minutes. The slides are then placed in 2 x SCC solution for 30 minutes with gentle rocking, then in 1 x SCC for 30 minutes with gentle rocking. Fluoresceinated avidin (Vector, Burlingame, Calif.) is diluted 1:1000 (1 μg/ml). The back of the slide and around cell area are wiped. Two hundred microliters of the avidin-fluorescein is added to each slide and incubated in the box for 30 minutes at room temperature. The slides are rinsed sequentially in 1) 4 x SCC for 10 minutes with rocking, 2) 4 x SCC, 0.1% Tween-20 for 10 minutes without rocking, 3) 4 x SSC for 10 minutes with rocking. The back of the slide and around cell area is wiped off. Ten microliters of anti-fade plus propidium iodine (10 ml PBS, 100 mg p-Phenylene diamine, 90 ml glycerol, pH 8.0, 10 μg/ml propidium iodide) is added. The slides are covered with coverslips and stored at 4°C. The slides may be stored for several days if necessary. Target DNA may be observed under a microscope by the presence of fluorescence.

Example 6

Chromosomal Typing

The enriched cells are exposed to 1 μg of colcemid (Sigma, St. Louis, Mo.) for one hour at 37°C. A hypotonic solution (0.075 M KCl) is added to the cells and incubated for 12 minutes at 37°C. The tubes are inverted once during the incubation to keep the cells suspended. Twenty drops of freshly prepared fixative (3:1 methanol:acetic acid) is added to the cells, vortexed, and then centrifuged for 8 minutes at approximately 250 g. Fresh fixative is added to the cells, followed by incubation for 1 hour at room temperature. The cells are centrifuged for 8 minutes at approximately 250 g and fresh fixative is added again. This process is repeated one more time. Finally, the cells are resuspended in a small amount of fixative and placed at 4°C overnight. The next day the cells are vortexed and placed onto microscope slides (Baxter, McGaw Park, Ill.) which have been cleaned with ethanol and dipped in distilled water. The slides are allowed to air dry for about 3-4 days, and then are treated with 0.005% trypsin (Difco Bactotrypsin, VWR Scientific, San Francisco, Calif.) for 30 to 35 seconds. The

slides are washed twice in PBS Plus 1% FBS, followed by washing in PBS only. The cells are stained with Wright's solution (Sigma Chemical Co., St. Louis, Mo.), followed by two washes with deionized water. The slides are scanned for evidence of metaphase cells and typed by conventional cytogenetics.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) removing unbound blood products; and

(c) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

2. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) removing unbound blood products;

(c) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and

(d) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

3. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) removing unbound blood products;

(c) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells;

(d) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs; and

(e) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

4. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of the maternal blood with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(b) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs;

(c) incubating cells remaining after hemolysis with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells; and

(d) removing unbound blood products.

5. The method of claim 4, including, subsequent to removing said unbound blood products, incubating the cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

6. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of the maternal blood in the presence of erythropoietin such that the fetal cells are enriched;

(b) incubating the enriched cells with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells; and

(c) removing unbound blood products.

7. The method of claim 6, including, subsequent to removing said unbound blood products, incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and

incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

8. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood in the presence of erythropoietin such that the fetal cells are enriched;

(b) incubating the erythropoietin enriched cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(c) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs;

(d) incubating the enriched cells from step (c) with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells; and

(e) removing unbound blood products.

9. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(b) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs;

(c) incubating the enriched cells from step (b) in the presence of erythropoietin such that the fetal cells are enriched;

(d) incubating the erythropoietin enriched cells with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells; and

(e) removing unbound blood products.

10. The method of claims 1 through 9 wherein said ligand is selected from the group consisting essentially of antibodies, erythropoietin, and transferrin.

11. The method of claims 1 through 9, including, subsequent to the step of removing said unbound blood products, releasing the immobilized cells.

12. The method of claim 11 wherein said cells are released by mechanical agitation.

13. The method of claims 1 through 9 wherein the step of removing comprises rinsing the bound cells with a physiological solution.

14. The method of claim 13 wherein said physiological solution is selected from the group consisting of PBS, PBS plus albumin, normal saline, and cell culture media.

15. The method of claims 1 through 9, including, subsequent to the step of preferentially enriching the fetal cells, incubating the enriched cells with a marker capable of selectively binding to said fetal cells.

16. The method of claims 1 through 9 wherein said ligand is immobilized on a solid support selected from the group consisting of hollow fibers, beads, magnetic beads, plates, dishes, flasks, meshes, screens, solid fibers, membranes, and dipsticks.

17. The method of claims 2, 3, 4, 7, 8, or 9 wherein said carbonic anhydrase inhibitor is selected from the group consisting of sulfanilamide and acetazolamide.

18. The method of claims 2, 3, 4, 7, 8, or 9 wherein said chloride ions are selected from the group consisting of NaCl, KCl, $MgCl_2$, and $CaCl_2$.

19. The method of claims 2, 3, 4, 7, 8, or 9 wherein said ammonia is selected from the group consisting of NH_4Cl and ammonium salts.

20. The method of claims 2, 3, 4, 7, 8, or 9 wherein said carbon dioxide is selected from a group consisting of carbon dioxide in solution, carbonate, and bicarbonate.

21. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a first member chemically linked to a ligand capable of specifically binding to fetal nucleated, erythroid

cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(c) removing unbound blood products; and

(d) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

22. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a first member chemically linked to a ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(c) removing unbound blood products;

(d) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and

(e) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

23. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a first member chemically linked to a ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(c) removing unbound blood products;

(d) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells;

(e) incubating the cells containing accumulated ammonium ions in the presence of ammonia ions and carbon dioxide such that selective hemolysis of maternal blood cells occurs; and

(f) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

24. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of the maternal blood with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(b) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs;

(c) incubating cells remaining after hemolysis with a first member chemically linked to a ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(d) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(e) removing unbound blood products.

25. The method of claim 24, including, subsequent to removing said unbound blood products, incubating the cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

26. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of the maternal blood in the presence of erythropoietin such that the fetal cells are enriched;

(b) incubating the enriched cells with a first member chemically linked to a ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(c) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(d) removing unbound blood products.

27. The method of claim 26, including, subsequent to removing said unbound blood products, incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and

incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

28. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood in the presence of erythropoietin such that the fetal cells are enriched;

(b) incubating the erythropoietin enriched cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(c) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs;

(d) incubating the enriched cells from step (c) with a first member chemically linked to a ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(e) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(f) removing unbound blood products.

29. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(b) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs;

(c) incubating the enriched cells from step (b) in the presence of erythropoietin such that the fetal cells are enriched;

(d) incubating erythropoietin enriched cells with a first member chemically linked to a ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(e) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(f) removing unbound blood products.

30. The method of claims 21 through 29 wherein said ligand is selected from the group consisting essentially of antibodies, erythropoietin, and transferrin.

31. The method of claims 21 through 29, including, subsequent to the step of removing said unbound blood products, releasing the immobilized cells.

32. The method of claim 31 wherein said cells are released by mechanical agitation.

33. The method of claims 21 through 29 wherein the step of removing comprises rinsing the bound cells with a physiological solution.

34. The method of claim 33 wherein said physiological solution is selected from the group consisting of PBS, PBS plus albumin, normal saline, and cell culture media.

35. The method of claims 21 through 29, including, subsequent to the step of preferentially enriching the fetal cells, incubating the enriched cells with a marker capable of selectively binding to said fetal cells.

36. The method of claims 21 through 29 wherein said second member is immobilized on a solid support selected from the group consisting of hollow fibers,

beads, magnetic beads, plates, dishes, flasks, meshes, screens, solid fibers, membranes, and dipsticks.

37. The method of claims 22, 23, 24, 27, 28, or 29 wherein said carbonic anhydrase inhibitor is selected from the group consisting of sulfanilamide and acetazolamide.

38. The method of claims 22, 23, 24, 27, 28, or 29 wherein said chloride ions are selected from the group consisting of NaCl, KCl, $MgCl_2$, and $CaCl_2$.

39. The method of claims 22, 23, 24, 27, 28, or 29 wherein said ammonia is selected from the group consisting of NH_4Cl and ammonium salts.

40. The method of claims 22, 23, 24, 27, 28, or 29 wherein said carbon dioxide is selected from a group consisting of carbon dioxide in solution, carbonate, and bicarbonate.

41. The method of claims 21 through 29 wherein the first member-second member binding pair are selected from the group consisting of biotin-avidin, biotin-streptavidin, biocytin-avidin, biocytin-streptavidin, methotrexate-dihydrofolate reductase, 5-fluorouracil-thimydylate synthetase, and riboflavin-riboflavin binding protein.

42. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a first ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said first ligand to the cells;

(b) incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand;

(c) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(d) removing unbound blood products; and

(e) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

43. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a first ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said first ligand to the cells;

(b) incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand;

(c) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(d) removing the blood products;

(e) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and

(f) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

44. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with a first ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said first ligand to the cells;

(b) incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand;

(c) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(d) removing unbound blood products;

(e) incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells;

(f) incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of other than fetal cells occurs; and

(g) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

45. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of the maternal blood with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(b) incubating the cells containing accumulated ammonium in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs;

(c) incubating the cells remaining after hemolysis with a first ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said first ligand to the cells;

(d) incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand;

(e) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(f) removing the blood products such that fetal cells are enriched.

46. The method of claim 45, including, subsequent to removing said unbound blood products, incubating the cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

47. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of the maternal blood in the presence of erythropoietin such that the fetal cells are enriched;

(b) incubating the enriched cells with a first ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow binding of said first ligand to the fetal nucleated, erythroid cells;

(c) incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand;

(d) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(e) removing unbound blood products.

48. The method of claim 47, including, subsequent to removing said unbound blood products, incubating the bound cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the bound cells; and

incubating the cells containing accumulated ammonium ions in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

49. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood in the presence of erythropoietin such that the fetal cells are enriched;

(b) incubating a sample of blood with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(c) incubating the enriched cells with a first ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow specific binding of said first ligand to the cells;

(d) incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to the first ligand;

(e) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(f) removing unbound blood products.

50. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within said cells;

(b) incubating the enriched cells with a first ligand capable of specifically binding to said cells under conditions and for a time sufficient to allow specific binding of said first ligand to the cells;

(c) incubating the enriched cells from step (b) in the presence of erythropoietin such that the fetal cells are enriched;

(d) incubating the sample with a first member chemically linked to a second ligand capable of specifically binding to the first ligand under conditions and for a time sufficient to allow the second ligand to bind to a first ligand;

(e) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1}; and

(f) removing unbound blood products.

51. The method of claims 42 through 50 wherein said ligand is selected from the group consisting essentially of antibodies, erythropoietin, and transferrin.

52. The method of claims 42 through 50, including, subsequent to the step of removing said unbound blood products, releasing the immobilized cells.

53. The method of claim 52 wherein said cells are released by mechanical agitation.

54. The method of claims 42 through 50 wherein the step of removing comprises rinsing the bound cells with a physiological solution.

55. The method of claim 54 wherein said physiological solution is selected from the group consisting of PBS, PBS plus albumin, normal saline, and cell culture media.

56. The method of claims 42 through 50, including, subsequent to the step of preferentially enriching the fetal cells, incubating the enriched cells with a marker capable of selectively binding to said fetal cells.

57. The method of claims 42 through 50 wherein said second member is immobilized on a solid support selected from the group consisting of hollow fibers, beads, magnetic beads, plates, dishes, flasks, meshes, screens, solid fibers, membranes, and dipsticks.

58. The method of claims 43, 44, 45, 48, 49, or 50 wherein said carbonic anhydrase inhibitor is selected from the group consisting of sulfanilamide and acetazolamide.

59. The method of claims 43, 44, 45, 48, 49, or 50 wherein said chloride ions are selected from the group consisting of NaCl, KCl, $MgCl_2$, and $CaCl_2$.

60. The method of claims 43, 44, 45, 48, 49, or 50 wherein said ammonia is selected from the group consisting of NH_4Cl and ammonium salts.

61. The method of claims 43, 44, 45, 48, 49, or 50 wherein said carbon dioxide is selected from a group consisting of carbon dioxide in solution, carbonate, and bicarbonate.

62. The method of claims 42 through 50 wherein the first member-second member binding pairs are selected from the group consisting of biotin-avidin, biotin-streptavidin, biocytin-avidin, biocytin-streptavidin, methotrexate-dihydrofolate reductase, 5-fluorouracil-thimydylate synthetase, and, riboflavin-riboflavin binding protein.

63. The method of claims 42 through 50 wherein said second ligand is an antibody.

64. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with biotinylated anti-erythroblast antibody under conditions and for a time sufficient to allow the antibody to bind to the fetal nucleated, erythroid cells;

(b) passing the cells through a packed bed of porous material having avidin bound to the surface thereof so as to adsorb the biotinylated antibody to the avidin on the surface of the porous material, thereby binding the fetal cells and adult cells to the porous material;

(c) rinsing the packed bed to remove unbound blood products;

(d) releasing bound cells by mechanical agitation of the packed bed; and

(e) incubating the released cells in the presence of erythropoietin such that the fetal cells are preferentially enriched.

65. A method for enriching fetal nucleated, erythroid cells from maternal blood, comprising:

(a) incubating a sample of maternal blood with biotinylated anti-erythroblast antibody under conditions and for a time sufficient to allow the antibody to bind to the fetal nucleated, erythroid cells;

(b) passing the cells through a packed bed of porous material having avidin bound to the surface thereof so as to adsorb the biotinylated antibody to the avidin on the surface of the porous material, thereby binding the fetal cells and adult cells to the porous material;

(c) rinsing the packed bed to remove unbound blood products;

(d) releasing bound cells by mechanical agitation of the packed bed;

(e) incubating the released cells with ammonia and chloride ions and a carbonic anhydrase inhibitor under conditions and for a time sufficient to allow accumulation of ammonium ions within the released cells; and

(f) incubating the released cells in the presence of ammonia and carbon dioxide such that selective hemolysis of maternal blood cells occurs.

66. A method for enriching fetal nucleated, erythroid cells from maternal blood, and for detecting the presence of a selected genetic material comprising:

(a) incubating a sample of maternal blood with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) removing unbound blood products;

(c) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched;

(d) exposing the genetic material associated with said preferentially enriched cells;

(e) incubating the genetic material associated with the fetal cells with a labelled probe capable of specifically hybridizing to said genetic material under conditions and for a time sufficient to allow hybridization to occur; and

(f) detecting the presence of the hybridized labelled probe.

67. The method of claim 66, including, subsequent to the step of exposing the genetic material, denaturing the genetic material.

68. A method for enriching fetal nucleated, erythroid cells from maternal blood, and for typing chromosomes from said cells comprising:

(a) incubating a sample of maternal blood with an immobilized ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) removing unbound blood products;

(c) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched; and

(d) typing the chromosomes associated with the enriched fetal cells.

69. A method for enriching fetal nucleated, erythroid cells from maternal blood, and for detecting the presence of a selected genetic material comprising:

(a) incubating a sample of maternal blood with a first member chemically linked to a ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(c) removing unbound blood products;

(d) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched;

(e) exposing the genetic material associated with said preferentially enriched cells;

(f) incubating the genetic material associated with the fetal cells with a labelled probe capable of specifically hybridizing to said genetic material under conditions and for a time sufficient to allow hybridization to occur; and

(g) detecting the presence of the hybridized labelled probe.

70. The method of claim 69, including, subsequent to the step of exposing the genetic material, denaturing the genetic material.

71. A method for enriching fetal nucleated, erythroid cells from maternal blood, and for typing chromosomes from said cells comprising:

(a) incubating a sample of maternal blood with a first member chemically linked to a ligand capable of specifically binding to fetal nucleated, erythroid cells under conditions and for a time sufficient to allow specific binding of said ligand to the cells;

(b) adsorbing the cells to an immobilized second member, said second member capable of binding to the first member with an affinity constant of greater than about 10^8 M^{-1};

(c) removing unbound blood products;

(d) incubating the bound cells in the presence of erythropoietin such that the fetal cells are preferentially enriched; and

(e) typing the chromosomes associated with the enriched fetal cells.

72. A method for typing chromosomes of fetal nucleated erythroid cells, comprising:

(a) incubating the fetal nucleated erythroid cells in a media containing erythropoietin under conditions and for a time sufficient to induce metaphase in said cells;

(b) fixing the DNA of said cells;

(c) staining the fixed DNA such that chromosomes may be observed; and

(d) examining the stained DNA thereby allowing the typing of the chromosomes.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02789

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 Q 1/24 G 01 N 33/569 C 12 N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 Q G 01 N C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	BLOOD, vol. 73, no. 1, January 1989, Grune & Stratton, Inc., E. FIBACH et al.: "Proliferation and maturation of human erythroid progenitors in liquid culture", pages 100-103, see the abstract; page 102, lines 12-13 ---	1,21,26 ,42,47, 64,66-72
Y	BLOOD, vol. 47, no. 6, June 1976, Grune & Stratton, Inc., S.H. BOYER et al.: "Enrichment of erythrocytes of fetal origin from adult-fetal blood mixtures via selective hemolysis of adult blood cells: An aid to antenatal diagnosis of hemoglobinopahties", pages 883-897, see the abstract ---	2,4,17-20,22, 24,29, 43,45, 49,65
A	US,A,4675286 (E. CALENOFF) 23 June 1987, see the abstract; column 3, line 10 - column 7, line 42; column 10, line 25 - column 11, line 14 --- -/-	1,2,11-16

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23-08-1991	03.10.91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme. M. van der Drift

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF IMMUNOLOGICAL METHODS, vol. 91, 1986, Elsevier Science Publishers B.V. (Biomedical Division), R.J. BERENSON et al.: "Positive selection of viable cell populations using avidin-biotin immunoadsorption", pages 11-19, see the abstract ---	1,41
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 76, no. 3, March 1979, L.A. HERZENBERG et al.: "Fetal cells in the blood of pregnant women: Detection and enrichment by fluorescence-activated cell sorting", pages 1453-1455, see the whole article ---	
A	BLOOD, vol. 69, no. 1, January 1987, M.R. LOKEN et al.: "Flow cytometric analysis of human bone marrow: I. Normal erythroid development", pages 255-263, see the abstract ---	10
Y	DE,A,3044015 (U. CLAUSSEN) 3 June 1982, see claim 1; page 5, line 1 - page 7, line 14 ---	72
P,Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 87, no. 9, 4 May 1990, D.W. BIANCHI et al.: "Isolation of fetal DNA from nucleated erythrocytes in maternal blood", pages 3279-3283, see the whole article ---	1,2,4, 17-22, 24,26, 29,42, 43,45, 47,49
P,Y	---	64-71
E	WO,A,9107660 (CHILDREN'S MEDICAL CENTER CORP.) 30 May 1991, see the whole document, especially page 11, line 8 - page 14, line 9; page 23, line 16 - page 26, line 6 -----	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9102789 SA 47755

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 20/09/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4675286	23-06-87	None	
DE-A- 3044015	03-06-82	None	
WO-A- 9107660	30-05-91	None	

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82